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PII: S2213-7165(19)30149-3
DOI: https://doi.org/10.1016/j.jgar.2019.06.006
Reference: JGAR 962

To appear in:

Received date: 1 March 2019
Revised date: 5 June 2019
Accepted date: 6 June 2019

Please cite this article as: Monteiro LM, Löbenberg R, Fotaki N, de Araújo GLB, Cotrim PC, Bou-Chacra N, Co-delivery of buparvaquone and polymyxin B in nanostructured lipid carrier for leishmaniasis treatment, *Journal of Global Antimicrobial Resistance* (2019), https://doi.org/10.1016/j.jgar.2019.06.006

Co-delivery of buparvaquone and polymyxin B in nanostructured lipid carrier for leishmaniasis treatment

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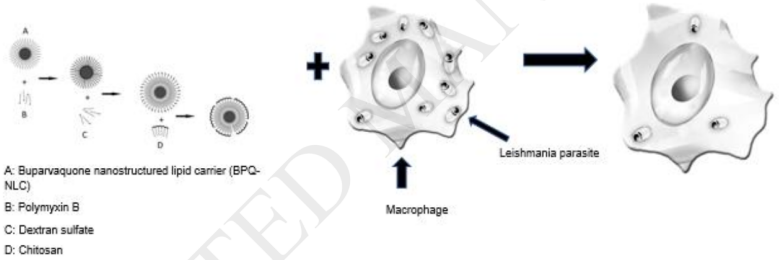
Graphical abstract



Highlights

- The low water-soluble drug buparvaquone was successfully encapsulated in nanostructured lipid carrier by high-pressure homogenization.

- The co-encapsulation of buparvaquone as the main drug, and polymyxin B as an adjuvant drug were possible using nanostructured lipid carrier formulation.
- Biopolymers chitosan and dextran coating were used for nanoparticles coating as moieties for macrophage-targeted delivery.
- Cationic and anionic nanoparticles were prepared using the biopolymers.
- *In vitro* cytotoxicity test revealed the formulation safety, even for cationic nanoparticles.
- *In vitro* leishmanicidal test against amastigotes of *L.chagasi* showed increased activity of nanoparticles compared to free drug, up to 3.0-fold.

Abstract

Objectives

The objective of this study was to describe the preparation and in vitro evaluation of the surface modified nanostructured lipid carrier (NLC) using chitosan and dextran in the co-delivery of buparvaquone (BPQ) and polymyxin B (PB) against leishmaniasis.

Methods

NLC was prepared using high high-pressure homogenization; polymyxin B binding and surface modifications with biopolymers were achieved by electrostatic interaction. *In vitro* cytotoxicity was assessed in mouse peritoneal macrophages and leishmanicidal activity, in amastigotes of *L. infantum*.

Results

The performance attributes of BPQ-NLC, BPQ-NLC-PB[A^-] (anionic) and BPQ-NLC-PB[C^+] (cationic) were respectively: Z-average of 173.9±1.6, 183.8±4.5 and 208.8±2.6 nm; zeta potential of -19.6±1.5, -20.1±1.1 and +31.1±0.8 mV; CC_{50} of 583.4±0.10, 203.1±0.04 and 5.7±0.06 μM; IC_{50} of 229.0±0.04, 145.7±0.04 and 150.5±0.02 nM. NLCs *in vitro* leishmanicidal activity showed up to 3.1-fold increase when comparing with free BPQ ($p < 0.05$, $\alpha = 0.05$).

Conclusions

The developed NLC proved to be a promising formulation to overcome the drawbacks of current leishmaniasis treatment by the co-delivery of two alternative drugs and macrophage targeting modified surface.

Keywords: Buparvaquone, Nanostructured Lipid Carrier, Leishmaniasis, Polymyxin B, Dextran, Chitosan.

1. Introduction

Leishmania parasite is the etiologic agent of leishmaniasis, and its intracellular location hinders the access of drugs[1]. Therefore, the treatment requires large and repeated doses, which rouses toxic effects and parasite resistance[2]. Buparvaquone (BPQ) was tested for the first time in 1992 by Croft and colleagues against *L.donovani*. However, due to the poor water solubility of <300 ng.mL^{-1} (value below the limit of quantification determined by HPLC), BPQ showed low *in vivo* activity[3].

Various strategies have been used to overcome these limitations. One of the most successful ones refers to the nano-based drug delivery approach, which can increase the intracellular concentration of leishmanicidal drugs, e.g., nanostructured lipid carrier (NLC)[4]. These delivery systems can improve the therapy by releasing drugs substances to the site of action, as a result of their preferential internalization by macrophages[2]. Moreover, NLC can increase the water solubility of drugs, such as BPQ.

A second approach is the use of two or more drugs substances with different pharmacological mechanisms. This combination can provide a synergistic effect by increasing therapeutic efficacy and reducing the risk of parasite resistance. In this case, antimicrobial peptides (AMP) are promising compounds. Among the AMPs, polymyxin B can enhance macrophage cell permeability and activate biochemical cell alterations and apoptosis.[5]

Additionally, a third approach is the use of biopolymers, such as chitosan and dextran. These molecules are recognized by SIGN-1 and mannan receptors (MR) from macrophages, which can induce nanoparticle phagocytosis and immune responses[6,7].

Taking these premises into account, this study describes the preparation and *in vitro* evaluation of a surface modified NLC using chitosan and dextran in the co-delivery of BPQ and polymyxin B for leishmaniasis treatment.

2. Materials and methods

2.1. Materials

Softisan 154 (hydrogenated palm oil) was donated by Gattefossé (France) and medium chain triglycerides (MCT) by Abitec (USA). Buparvaquone (BPQ) (99.7% purity by HPLC) was purchased from Uniwise (China). Kolliphor P188 (poloxamer 188) was acquired from BASF (Germany). Polymyxin B (PB) was purchased from Biotika (Slovakia) (8106 units.mg^{-1}) and chitosan from NovaMatrix (Norway). Dextran sulfate sodium salt (Mr ~4000) and DEAE-dextran hydrochloride were purchased from MilliporeSigma (Germany). All other chemicals used were of at least analytical grade.

2.2. Analytical method for quantification of BPQ by HPLC

A method described by Monteiro and colleagues[8] was applied. The conditions were: Xterra column, C8 100x4.6 mm, 3.5µm (Waters Corporation, USA). The mobile phase of glacial acetic acid (1% v/v), acetonitrile and methanol (25:65:10), 35°C, 50 µL injection, and UV λ=252 nm.

2.3. Preparation of BPQ-NLC, BPQ-NLC-PB-[A$^-$] and BPQ-NLC-PB-[C$^+$]

BPQ-NLC formulations were prepared as previously described[8]. Briefly, the heated lipid phase was dispersed in the aqueous phase (70 °C). Pre-homogenization was performed using a high-performance disperser (8,000 RPM for 5 min) (T25 digital ULTRA-TURRAX, IKA, Staufen, Germany). Afterward, the coarse emulsion was passed through a high-pressure

homogenizer (Nano DeBEE 45-2, Bee International, South Easton, MA, USA) at 600 bars for five cycles.

The solid lipid, liquid lipid, and the surfactant were Softisan 154 (1.7% w/w), MCT (3.3% w/w) and Kolliphor P188 (4.0% w/w), respectively. BPQ drug loading was 1.3% w/w of lipid phase due to drug solubility found in the pre-formulation tests[8].

Briefly, the co-delivered BPQ-PB in NLC was prepared by the addition of PB stock solution (final concentration of 3000 UI.mL^{-1}) to BPQ-NLC, under magnetic stirring (100 rpm, 1h). The decrease in the zeta potential showed the PB binding to BPQ-NLC surface by electrostatic interaction.

A similar method was used for the preparation of the coated nanoparticles (anionic [A$^-$] and a cationic [C$^+$] nanoparticles) of BPQ-NLC-PB and the biopolymers. For the anionic formulation (BPQ-NLC-PB-[A$^-$]) a chitosan stock solution was added to BPQ-NLC-PB, following the addition of dextran sulfate stock solution up to 0.55% w/v, for an additional hour. For the cationic formulation (BPQ-NLC-PB-[C$^+$]), a DEAE-dextran stock solution was added to BPQ-NLC-PB to reach a final concentration of 0.05% w/v.

2.4. Z-average, polydispersity index (PDI) and zeta potential (ZP)

The Z-average and PDI were performed by photon correlation spectroscopy (PCS) and ZP, by electrophoretic mobility. For both methods, Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) was used. The measurements (n=3) were carried out with purified water (conductivity of 70 µS.cm^{-1}, pH 6.5 ± 0.2, and 25 °C).

2.5. Thermal analysis by differential scanning calorimetry (DSC) and thermogravimetry analysis (TG)

TG analysis of dried nanoparticles and free BPQ were performed using 7020 and TG/DTA A72000 (SII NanoTechnology, Japan). Samples of 5 mg were heated from 25 to 600 °C at a rate of 10°C min^{-1} in a nitrogen atmosphere (50 mL.min^{-1}). DSC curves were acquired

using EXSTAR DSC 7020 (SII NanoTechnology, Japan). Samples of 5 mg were heated from 25 to 350°C at a rate of 10°C min^{-1} in a nitrogen atmosphere (50 $mL.min^{-1}$).

2.6. Nanoparticle morphology by transmission electron microscopy (TEM)

Images were acquired using a Morgagni 268 transmission electron microscope (Philips/FEI, Oregon, USA). The diluted samples (1:20) were placed over regular TEM grids, and the background was stained with phosphotungstic acid 10 % w/v.

2.7. *In vitro* cytotoxicity and leishmanicidal activity against *L. infantum* intracellular amastigotes

In vitro cytotoxicity and leishmanicidal activity were performed as described previously[9], using an established protocol from the Institute of Tropical Medicine of the University of São Paulo. Briefly for *in vitro* cytotoxicity: macrophages ($1x10^6$) were incubated (37°C, 24h, 24 wells plate) with increased concentrations of free BPQ and NLC formulations (n=6). Afterward, chromogenic MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution ($5mg.mL^{-1}$) was added and the optical density was recorded using Multiskan™ GO microplate spectrophotometer (Fisher Scientific, UK) (λ= 595 nm).

Briefly, for intracellular amastigote test: *L. infantum* infected macrophages ($1x10^6$) were incubated (37°C, 24h) with increased concentrations of free BPQ and NLC formulations (n=6). The cells were Giemsa stained, and the number of infected, uninfected cells (n=200) and amastigotes per cell were counted by microscopic examination. IC_{50} and CC_{50} were calculated by non-linear regression using GraphPad Prism 5.01. Statistical analyses were performed using ANOVA with a significance level of 0.05 and Tukey's multiple comparison test.

3. Results and discussion

3.1. Preparation of BPQ-NLC, BPQ-NLC-PB, BPQ-NLC-PB-[A^-] and BPQ-NLC-PB-[C^+]

BPQ-NLC showed Z-average of 173.9±1.6nm and PDI of 0.164±0.005, which showed a homogenous and monomodal particle size distribution. ZP, BPQ loading and %EE were: +19.6±1.5 mV, 0.5054±0.0108 mg.mL^{-1} and 99.72±0.33%, respectively. These values showed the suitability of high-pressure homogenization for NLC preparation.

BPQ-NLC-PB showed Z-average of 171.9±1.6 nm and monomodal particle size distribution. PDI and ZP values were -3.7±0.5 mV and 0.110±0.003, respectively. Table 1 shows the physicochemical attributes of BPQ-NLC-PB-[A$^-$] and BPQ-NLC-PB-[C$^+$]. Both formulations held the nanometric range, monomodal distribution, and low PDI (<0.3).

The BPQ loading was limited by its solubility in lipids[8]. NLC is characterized by the drug substance dissolution inside the lipid matrix, which prevent drug expulsion and protect it from degradation. Thus, surfactants are not used for drug solubilization; their hole is primarily nanoparticle stabilization. Therefore, NLC can be prepared with low surfactant content when compared to self-emulsifying drug delivery systems (SEDDS). These formulations have the potential for the development of a commercial product due to their high leishmanicidal activity (IC50), which provided the suitability of low drug loading NLC.

3.2. Thermal analysis by differential scanning calorimetry (DSC) and thermogravimetry analysis (TG)

The DSC curves showed the reduction of BPQ melting peak (185°C) when encapsulated in NLC. The decrease in the drug crystallinity (amorphization) is an indicator of drug solubilization in the lipid matrix, corroborating the %EE values. The TG curves revealed no weight loss up to 250°C, which shows no drug or NLC degradation during the high-pressure homogenization.

3.3. Nanoparticles morphology by TEM

Figure 1 shows the microscopy of the developed formulations. TEM revealed the sphericity and the absence of agglomerated particles and size distribution compatible with the PCS results.

3.4. In vitro cytotoxicity and leishmanicidal activity in *L. infantum* intracellular amastigotes

Table 2 shows the CC_{50} and IC_{50} of free BPQ and formulations. BPQ-NLC showed cytotoxicity comparable to BPQ, CC_{50} of 583.4±0.10 μM and 524.4±0.16 μM, respectively. The anionic formulation (BPQ-NLC-PB-[A^-]) showed an approximately 2.5-fold increase in the toxicity (CC_{50}: 203.1±0.04 μM). This increase was due to the PB addition, which showed a CC_{50} of 83.7±0.04 μM (Table 2), as well as BPQ-NLC-PB-[C^+], which showed increased cytotoxicity (CC_{50}: 5.7±0.06) due to the positive charge.

Cationic nanoparticles (BPQ-NLC-PB-[C^+]) showed CC_{50} 9.2-fold lower (5.7±0.06 μM) than BPQ-NLC. The decreased value is explained by the interaction between cells and the nanoparticles charges. Cationic nanoparticles led to pronounced disruption in the cell membrane integrity, increasing the membrane permeability and further death from apoptosis[12, 13]. On the other hand, these interactions trigger the release of pro-inflammatory response by inducing Th1 cytokines expression[6]. This mechanism can be a valuable tool to parasite elimination since one of the main mechanisms of Leishmania survival is the innate immune response modulation[2].

The cytotoxicity of the controls without BPQ, NLC, NLC-PB-[A^-], NLC-PB-[C^+], the CC_{50} were 626.5±0.8, 233.9±0.27, and 8.7±0.05 μM, respectively. These values were close to their respective formulations with BPQ (Table 2). Therefore, the BPQ did not increase the cytotoxicity of the developed NLCs.

The BPQ-NLC showed IC_{50} 2-fold (229.0±0.04 nM) lower than the free BPQ (456.5±0.05 nM). BPQ-NLC-PB-[A^-] and BPQ-NLC-PB-[C^+] revealed improved and similar activity, 145.7±0.02 and 150.5±0.02 nM, respectively, which is an increase of 3.0 and 3.1-fold when compared to free BPQ. The comparisons among all IC_{50} revealed p-value <0.05 (α=0.05) and their distinctiveness. The controls without BPQ showed IC_{50} increased values (Table 2), which indicated that the improved activities of the developed formulations were due to modified action of the drug by the structure of the nanoparticle.

The BPQ-NLC without coating increased the selectivity index (SI) of BPQ from 1148.7 to 2547.6 μM (2.2-fold). Similarly, when the drug was present, the coated formulations showed increased SI values up to 64-fold (Table 2).

However, inconsistent values of free BPQ IC_{50} were found in three previous studies[3, 10, 11]. One of them tested BPQ against *L. donovani*. Another study used a strain of *L. infantum* isolated from dogs and the third used *L. infantum* isolated from human hostage. The values diverged from <15 to 1500 nM.

Moreover, different methodologies were used. A number of cells per well ranged from 5×10^4 to 1×10^6. Time of incubation varied from 24 hours to seven days. Quantification methods were also discrepant, two of them used Giemsa-stained counting, and one used a fluorometric-based method. Thus, no direct comparison of the results was feasible.

Nevertheless, free drug and nanostructured formulations leishmanicidal activities comparisons were possible, showing the relevance of the nano-based drug delivery approach and their potential for improving leishmaniasis treatment.

4. Conclusions

This study showed the combination of three strategies to increase BPQ leishmanicidal activity which include the use of lipid nanoparticles as a low water-solubility drug carrier, BPQ and polymyxin B combination and the use of biopolymers in macrophage targeting drug delivery. The developed formulations revealed suitable physicochemical attributes and increased *in vitro* leishmanicidal activity when compared with free BPQ while upholding low cytotoxicity. Therefore, they are promising candidates for further *in vivo* and immunological tests.

Furthermore, the developed NLCs can be used as a platform for the development of oral and parenteral products, which can contribute to overcoming the drawbacks of conventional leishmaniasis treatment.

Acknowledgements

The paper was revised by Jim Hesson (http://academicenglishsolutions.com/AES/home.html).

Declarations

Funding: This study was supported by the National Council for Scientific and Technological Development (CNPq-Brasil - 400455/2014-5). The authors thank CAPES (Coordination for the Improvement of Higher-Level Personnel) for the financial support for this research.

Competing Interests: No conflict of interest

Ethical Approval: Yes, from Institue of Tropical Medicine, CPE-IMT/000269A

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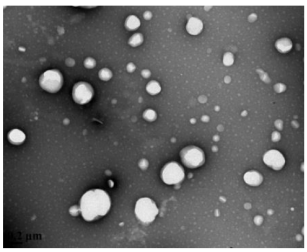
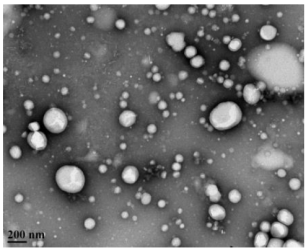
Figure 1





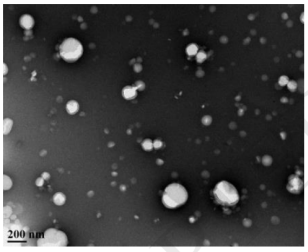


Table 1 - Z-average, PDI, ZP, BPQ drug loading and encapsulation efficiency of BPQ-NLC, BPQ-NLC-PB-[A-] and BPQ-NLC-PB-[C+] (n=3).

Formulation	Z-average (nm)	PDI	ZP (mV)	BPQ DL ($mg.mL^{-1}$)	BPQ EE (%w/v)
BPQ-NLC	172.3±1.8*	0.122±0.011	-30.9±1.7	0.495±0.003	99.3±0.7
NLC (blank)	169.0±1.2*	0.147±0.004	-31.4±1.3	n/a	n/a
BPQ-NLC-PB-[A^-]	183.8±4.5*	0.139±0.005	-20.1±1.1	0.460±0.006	99.5±0.5
NLC-PB-[A^-]	188.2±2.1*	0.148±0.023	-17.9±0.3	n/a	n/a
BPQ-NLC-PB-[C^+]	208.8± 2.6*	0.140±0.019	+29.7±0.2	0.497±0.003	99.7 ±0.5
NLC-PB-[C^+]	206.0±4.7*	0.090±0.002	+31.1±0.8	n/a	n/a

* monomodal distribution. BPQ: buparvaquone. PDI: polydispersity index. ZP: zeta potential. BPQ DL: BPQ drug loading. BPQ EE: BPQ encapsulation efficiency. BPQ-NLC-PB-[A^-]: BPQ nanostructured lipid carrier and polymyxin B coated with chitosan and dextran sulfate; BPQ-NLC-PB-[C^+]: BPQ nanostructured lipid carrier and polymyxin B coated with DEAE-dextran.

Table 21. CC_{50} and IC_{50} (95% IC) of buparvaquone (BPQ), BPQ nanostructured lipid carrier (BPQ-NLC), BPQ-NLC with polymyxin B (PB) coated with chitosan and dextran sulfate (BPQ-NLC-PB-[A^-]) and BPQ-NLC with PB coated with DEAE-dextran (BPQ-NLC-PB-[C+])

	CC_{50} (µM)	**IC_{50} (nM)**	**Selectivity Index**
Blank NLC*	626.5 (0.8 - 17265) SD: 0.80	51775 (29573 - 90647) SD: 0.11	6.3
NLC-PB-[A^-]*	233.9 (63.68 - 859.1) SD: 0.28	10827 (8734 - 13422) SD: 0.04	21.6
NLC-PB-[C+]*	8.7 (6.9 - 11.1) SD: 0.05	9278 (6466 - 13313) SD: 0.07	0.9
BPQ	524.4 (252.2 - 1090.0) SD: 0.16	456.5 (332.4 - 627.0) SD: 0.05	1148.7
Polymyxin B	83.7 (70.7 - 101.1) SD: 0.04	12620 (10570 - 14830) SD: 0.04	6.6
BPQ-NLC	583.4 (362.6 - 938.6) SD: 0.10	229.0 (190.5 - 275.2) SD: 0.04	2547. 6
BPQ-NLC-PB-[A^-]*	203.1 (169.6 - 243.3) SD: 0.04	145.7 (132.3 - 160.6) SD: 0.02	1394.0
BPQ-NLC-PB-[C+]*	5.7 (4.2 - 7.8) SD: 0.06	150.5 (139.5 - 162.4) SD: 0.021	37.9

* CC_{50} and IC_{50} concentrations from BPQ; n=6; SD= standard deviation